Three distinct clades of cultured heterocystous cyanobacteria constitute the dominant N_2-fixing members of biological soil crusts of the Colorado Plateau, USA

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Received 16 May 2006; revised 2 November 2006; accepted 3 November 2006.
First published online 19 January 2007.

DOI:10.1111/j.1574-6941.2006.00265.x

Editor: Karl Ritz

Keywords
cyanobacteria; nitrogen fixation; *nifH*; biological soil crust; *Nostoc*; *Scytonema*.

Abstract

The identity of the numerically dominant N_2-fixing bacteria in biological soil crusts of the Colorado Plateau region and two outlying areas was determined using multiple approaches, to link the environmental diversity of *nifH* gene sequences to cultured bacterial isolates from the regions. Of the *nifH* sequence-types detected in soil crusts of the Colorado Plateau, 89% (421/473) were most closely related to *nifH* signature sequences from cyanobacteria of the order *Nostocales*. N_2-fixing cyanobacterial strains were cultured from crusts and their morphotypes, 16S rRNA gene and *nifH* gene sequences were characterized. The numerically dominant diazotrophs in the Colorado Plateau crusts fell within three clades of heterocystous cyanobacteria. Two clades are well-represented by phylogenetically and morphologically coherent strains, corresponding to the descriptions of *Nostoc commune* and *Scytonema hyalinum*, which are widely recognized as important N_2-fixing components of soil crusts. A third, previously-overlooked clade was represented by a phylogenetically coherent but morphologically diverse group of strains that encompass the morphogenera *Tolypothrix* and *Spirirestis*. Many of the strains in each of these groups contained at least two *nifH* copies that represent different clusters in the *nifH* environmental survey.

Introduction

In many terrestrial environments, particularly semiarid and arid lands, cyanobacteria play a key role in soil development through the establishment and maintenance of biological soil crusts (simply referred to as crusts hereafter). Crusts are a complex assembly of bacteria, fungi, lichens, moss, and green algae that, together, cement soil particles in place and are major importers of carbon and nitrogen into the soil. Crusts also influence water retention and runoff, capture trace elements via aeolian deposition, decrease surface albedo, provide habitat for soil microfauna, and may provide benefits for plant growth (Belnap & Lange, 2003). Cyanobacteria provide functions essential to the crusts that include synthesizing the gelatinous sheaths that stabilize the soil and increase water retention (Brock, 1975; Belnap & Gardner, 1993), protecting the crust from excessive UV radiation by producing UV-screening pigments (Castenholz & Garcia-Pichel, 2000; Bowker *et al.*, 2002), and supplying carbon (Beymer & Klopatek, 1991) and nitrogen (Evans & Ehleringer, 1993) through photosynthesis and N_2-fixation.

Aside from water, nitrogen is considered the limiting resource for soil productivity in many semiarid and arid lands, including the Colorado Plateau of the southwest USA (Post *et al.*, 1985; West, 1991). The contribution of crusts to total soil nitrogen input in this region is thought to be substantial, as crusts can cover up to 70% of the landscape (Evans & Ehleringer, 1993; Belnap, 1995). From morphological and culture-based studies of crust microorganisms, it was determined that heterocystous cyanobacteria, such as *Nostoc* species, were major suppliers of fixed nitrogen to soil crusts of the Colorado Plateau (Harper & Marble, 1988; Garcia-Pichel & Belnap, 1996a; Belnap, 2002). However, nonheterocystous cyanobacteria that are present in the crusts and surface soils are also able to fix nitrogen (Garcia-Pichel *et al.*, 2001), and the contributions to nitrogen fixation by chemoheterotrophs or

photoheterotrophs has not been studied in detail. Thus, the identity of keystone N_2-fixing organisms has not yet been firmly established.

Biological N_2 fixation occurs via the bacterial enzyme nitrogenase, and oligonucleotide primers that recognize the known diversity of genes encoding for the reductase subunit of this enzyme (*nifH*) have been designed and successfully used to investigate N_2-fixing community structure in a variety of environments (Zehr & Capone, 1996; Zehr *et al.*, 2003). An environmental survey of the composition and abundance of *nifH* genes from two types of crusts (dark and light pigmented) of the Colorado Plateau and Chihuahuan Desert found that over 90% of the *nifH* sequences recovered were classified as *Nostocales*-types, suggesting that these heterocystous, cyanobacterial species are the numerically dominant diazotrophs in crusts from the region (Yeager *et al.*, 2004). At that time, there were few examples of *nifH* sequences from cultured terrestrial cyanobacteria available for reliable species identification, and taxonomic designations were not assigned to the crust-derived *nifH* sequences. Furthermore, problems associated with horizontal gene transfer and multiple gene copies can convolute *nifH*-based phylogenies, making it difficult to *precisely* assign an environmental *nifH* sequence to a corresponding microbial taxa at any phylogenetic level (Zehr & Capone, 1996; Choo *et al.*, 2003; Dedysh *et al.*, 2004).

Through ongoing research aimed at assessing the potential effects of climate change on the occurrence, composition, and function of biological soil crusts of the southwest USA, we have accumulated a larger data set of *nifH* sequences from multiple sites within the Colorado Plateau and two outlying areas. The goal of the study described here was to determine the identity of the species harboring the environmental *nifH* sequences, and to culture and characterize diazotrophic strains that were representative of the major *nifH* sequence-types observed in the Colorado Plateau. By pairing information from the large *nifH* environmental surveys with morphological characteristics and 16S rRNA and *nifH* gene analysis of the cultured diazotrophs, we provide a more inclusive and precise evaluation of the numerically dominant, N_2-fixing microorganisms in biological soil crusts of the Colorado Plateau. The information and strains obtained from the current study are of value in interpreting other molecular and microbiological analyses of N_2 fixation in soils.

Materials and methods

Site descriptions

Crusts for DNA extraction and culturing material were collected from six sites. Four sites were in the Colorado Plateau – two within the Island in the Sky region of Canyonlands National Park, UT (CP1, 38°35.08′N, 109°49.16′W; CP2, 38°35.12′N, 109°49.22′W) and two within the Needles region of Canyonlands National Park (CP3, 38°09.88′N, 109°39.42′W; CP4, 38°09.70′N, 109°48.13′W). Collection sites located outside of the Colorado Plateau included one in the foothills of the Sangre de Cristo Mountains near Santa Fe, NM (SC1, 35°45.14′N, 105°53.22′W; 430–480 km to the southeast of the Canyonlands sites) and one in the Jornada Experimental Range, in the Chihuahuan Desert of NM (JO1, 32°31.80′N, 106°43.41′W; 670–720 km southeast of the Canyonlands sites and ~370 km south of the Sangre de Cristo site). Macroscopic colonies of *Nostoc commune* were collected from the soil/crust surface of several study sites.

The Colorado Plateau (CP1-4) sites range in elevation from *c.* 1500 to 1800 m and experience moderately cold winters and summers with hot days and cool nights. The long-term average annual precipitation is 215 mm, with 35% of that received as summer monsoonal rains. Site SC1 is situated at 2390 m, experiences cold winters and summers with warm days and cool nights, and receives an average precipitation of 390 mm. Site JO1 is situated at 1315 m within a much warmer area that experiences dry summers and only mild winter frosts. The amount of annual precipitation at Site JO1 (240 mm) is similar to that of the CP sites and most occurs as summer rains. The vegetation at the CP1, CP4, and SC1 sites was predominately pinyon-juniper woodlands. The vegetation at Sites CP2 and CP3 were the grass, *Stipa hymenoides*, and the shrub, *Coleogyne ramosissima*. *Fluorensia cernua* (tarbrush) was the dominant plant at Site JO1. Soils from each of the sites are classified as follows: CP1 and CP2, Rizno series loamy mixed, calcareous; CP3 and CP4, Begay sandy loam, calcareous; SC1, Nyjack series sandy loam; JO1, Regan series clay loam.

Crust material at the CP1, CP4, SC1, and JO1 sites was collected by removing intact crust from the soil surface ($\leq$ 5 cm depth). Crust material from the CP2 and CP3 sites was collected with soil cores to a depth of 10 cm and each sample was a composite of at least 20 subsamples. Multiple crust samples (8–12) were collected from each of the Canyonlands and Jornada sites, covering an area of *c.* 1 acre at each site to capture the crust heterogeneity (Yeager *et al.*, 2004). DNA was extracted from all samples, and terminal restriction fragment length polymorphism was used to analyze the diversity of *nifH* amplicons obtained from each of the samples. Libraries of *nifH* clones were then generated from three to five of the samples, chosen to represent the diversity of *nifH* sequences from each site. For the Sangre de Cristo Mountain site (SC1), two samples were collected from soils *c.* 50 m apart and *nifH* clone libraries were generated from each.

Cyanobacteria culture conditions

Crust material ($\leq$ 1 g) was used to inoculate 500-mL flasks containing BG-11 or BG-11$^-$ liquid medium (ATCC

Medium 616) to culture cyanobacteria (BG-11 medium contains 1.5 g L^{-1} $NaNO_3$ as the only source of nitrogen other than N_2, BG-11$^-$ medium does not contain $NaNO_3$). The flasks were then incubated for 1–2 months at ambient temperatures in the laboratory near a window that received 2–3 h of direct sunlight daily. A liquid subsample (5–20 μL) or small amount of cyanobacterial cell material from these cyanobacterial enrichments was then streaked onto BG-11 agar plates, which were incubated as described above or at 30 °C under constant light. Alternatively, a small amount of crust material was spread directly onto BG-11$^-$ plates to enrich for N_2-fixing cyanobacteria. Cyanobacteria strains that exhibited growth after three or more successive transfers onto fresh BG-11$^-$ plates were deemed diazotrophic. Unicyanobacterial- and clonal (genetically uniform) cultures were obtained by dragging individual filaments over agarose-solidified BG-11$^-$ medium (Garcia-Pichel *et al.*, 1996b).

Microscopy

Cyanobacteria colonies growing on BG-11$^-$ agar plates were examined under a dissecting scope for colony morphology, pigmentation, and motility. For microscopy, small subsections of colonies were picked from agar plates with watchmaker's forceps or a 26-gauge needle and prepared as a wet mount. Photomicrography was performed with a Zeiss Axioplan 2 microscope/digital camera system using bright field illumination.

DNA extraction

DNA was extracted from crust samples and macroscopic *N. commune* colonies using a bead-beating-, SDS-based DNA extraction protocol previously described (Kuske *et al.*, 1998). TENS buffer (1 mL) and soil or tissue (0.5 g) were added to a bead-beating tube and incubated at 70 °C for 1 h. Samples were then disrupted by bead-beating for 3 min at room temperature followed by centrifugation for 10 min at 12 000 ***g***. The supernatant was collected, DNA was precipitated with sodium acetate/ethanol, pelleted by centrifugation for 10 min at 13 000 ***g***, air dried for 20 min, and suspended in 30 μL of dH_2O. Extracted DNA was purified using Sephadex G-200 spin columns.

To obtain amplifiable DNA from cyanobacteria cultures, a small sample (~10–50 μg) of cells was removed from BG-11$^-$ agar plates and placed in a 1.5-mL microcentrifuge tube containing ~500 μL of sterile dH_2O. The cells were crushed with a pestle fashioned from a plastic pipet tip, processed through two to three freeze (− 20 °C)/thaw (boiling) cycles, and briefly centrifuged. Subsamples of the supernatant (1–2 μL or 1–2 μL of a 1 : 10 dilution) were used as template for subsequent PCRs.

16S rRNA gene and nifH PCR

To obtain 16S rRNA gene sequences from cultured cyanobacteria, we employed either a total eubacterial or a cyanobacteria-specific PCR approach. Amplification of eubacterial 16S rRNA gene sequences was performed with the 'universal' primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 787R (5′-CTACCAGGGTATCTAAT-3′), as previously described (Kuske *et al.*, 1997). Amplification of cyanobacteria-specific 16S rRNA gene sequences was achieved using forward primer CYA359F (5′-GGGGAATYTTCCGCAATGGG-3′) and equimolar amounts of two reverse primers, CYA781RA (5′-GACTACTGGGGTATCTAATCCCATT-3′) and CYA781RB (5′-GACTACAGGGGTATCTAATCCCTTT-3′) (Nübel *et al.*, 1997). Template DNA (1–2 ng) was added to a reaction mixture (30 μL total volume) containing: 15 μL iQ SYBR Green Supermix (Bio-Rad; Hercules, CA), 13.5 pmol CYA359F and 6.9 pmol each of CYA781RB and CYA781RB. PCR amplification was carried out in 96-well PCR plates with a Bio-Rad iCycler as follows: 95 °C for 7 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s.

PCR amplification of *nifH* fragments from DNA was performed using a nested protocol (Yeager *et al.*, 2004).

Cloning, sequencing, and phylogenetic analysis

16S rRNA gene and *nifH* amplicons were purified from PCR mixes using agarose gel electrophoresis to separate the amplicons and the QIAquick gel extraction kit (QIAGEN, Inc., Chatsworth, CA) to isolate DNA embedded in the agarose. Clone libraries for sequencing were generated with the TOPO TA cloning kit and TOP10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad). Sequencing was performed by the JGI Sequencing Group at LANL using the M13 forward or reverse primer. Poor quality and chimeric sequences were precluded from phylogenetic analysis. Alignment of DNA sequences was performed using CLUSTAL X v1.81 and visually inspected with the BioEdit sequence alignment editor (Thompson *et al.*, 1997; Hall, 1999). Phylogenetic analysis of sequences was performed with MEGA version 3.1 software (Kumar *et al.*, 2004). The *nifH* DNA dendogram was constructed using the minimum-evolution function of MEGA with initial trees obtained by the neighbor-joining (NJ) method. Distances were calculated with the Kimura two-parameter algorithm with complete deletion of gaps and missing data.

Nucleotide sequence accession numbers

Sequences were deposited in GenBank with accession numbers DQ531669–DQ531695 (*nifH*) and DQ531696–DQ531706 (16S rRNA gene).

Fig. 1. Neighbor-joining tree of partial *nifH* sequences (310 bp). Sequences generated in this study are designated by (◆). GenBank accession numbers for environmental sequences and database sequences are in parentheses following the name. Accession numbers for sequences obtained from cultured isolates and macroscopic colonies of *N. commune* are listed in Table 2. Sources of *nifH* sequences include: N_2-fixing cyanobacteria cultured from crusts (●), macroscopic colonies of *N. commune* SC and CP (○), clones from crust *nifH* gene libraries (★), UTEX culture collection isolates (■), and elsewhere. Multiple *nifH* sequences obtained from the same isolate are delineated as copy1 or copy2. Representative sequences of the *nifH* clusters found in arid land crusts and listed in Table 1 are indicated by brackets.

Results

Environmental survey of crust nifH sequences

A total of 693 partial *nifH* sequences (310 bp) were analyzed from crust samples collected from four sites within the Colorado Plateau (CP1–CP4), a site within the foothills of the Sangre de Cristo Mountains, NM (SC1), and a site within the Chihuahuan Desert, NM (JO1). Of those, 89% were classified cyanobacteria-type *nifH* (Table 1). The majority of cyanobacterial *nifH* sequences (83–100%) retrieved from each of the CP and SC1 sampling sites were classified into one of five clusters: S1, S2, N1, N2, or T1 (Fig. 1). In contrast, 58% of the *nifH* sequences-types retrieved from Site JO1 were grouped into Clusters U1 and U2, which were site-specific; furthermore, not a single representative of the N1, N2, or T1 clusters was identified among the JO1 *nifH* sequences.

In addition to the cyanobacterial groups, *nifH* sequences presumably from the *Proteobacteria* were detected in the crusts from all sites. Three sequence types, most closely related to *Alpha-* and *Betaproteobacteria*-type *nifH*, comprised 79% (58/72) of the noncyanobacterial *nifH* sequences recovered from the crusts. All of the *Betaproteobacteria*-type *nifH* sequences ($n = 17$) were retrieved from Site SC1 and may represent a common diazotrophic member of the crusts of the Sangre de Cristo area or simply a sampling anomaly (i.e. the sequences originated from organisms associated with a very small root section or other unseen piece of plant debris). All of the *Alphaproteobacteria*-type *nifH* sequences identified from the CP and SC1 sites were highly similar to the *nifH* Cluster O sequence type identified in a previous crust survey (Yeager *et al.*, 2004). The *nifH* Cluster O sequences constituted 58% (38/65) of the noncyanobacterial *nifH* sequence total from the CP and SC1 sites and were detected at each of these sites.

To determine whether the *nifH* composition at the four CP sites were more similar to each other than to the two New Mexico sites, we reduced the number of sequences in each library to the lowest common number ($n = 71$) and calculated distance matrices using Euclidian and Manhattan distance metrics. The CP2 and CP3 sequence sets were the most similar (Euclidian distance = 24.5), and the JO1 library was the most different from the other libraries (maximum Euclidian distance 58.9). The SF1 library was similar to the four CP sites even though it was 430–480 km to the southeast. These results suggest that the geographic range of the dominant, N_2-fixing bacteria found in crusts of our Colorado Plateau study sites may extend to other regions of similar vegetation and climate in the southwest USA. In contrast, the most abundant *nifH* sequences (Clusters U1 and U2) obtained from the single Chihuahuan Desert site surveyed (JO1) were not found in crusts of the 'cool' desert sites.

Table 1. Enumeration and distribution of *nifH* sequence-types in crusts collected at various sites

	Number of sequences retrieved						
	Colorado Plateau				Sangre de Cristo Mts	Chihuahuan Desert	
nifH Cluster*	CP1†	CP2	CP3	CP4	SC1	JO1	Total
S1	21	5	6	37	29	29	127
S2	14	1	1	46	4	7	73
N1	11	93	27	3	18	0	152
N2	10	17	0	1	11	0	39
T1	70	17	18	23	7	0	135
T2	0	0	0	0	0	4	4
U1	0	0	0	0	0	13	13
U2	0	0	0	0	0	52	52
Other cyanobacterial *nifH* sequences	0	1	11	5	0	8	25
Other bacterial *nifH* sequences	18	6	8	3	30	8	73
Total	144	140	71	118	99	121	693

*Sequences (310 bp) were grouped into clusters based on visual inspection of alignments, distance data, and NJ trees. Sequences within each cluster were at least 95% similar (most were > 98% similar).

†CP1 & CP2, Island in the Sky region of Canyonlands National Park, UT, USA; CP3 & CP4, Needles region of Canyonlands National Park, UT, USA; SC1, Sangre de Cristo Mountains near Santa Fe, NM, USA; JO1, Jornada Research Range, Chihuahuan Desert, NM, USA.

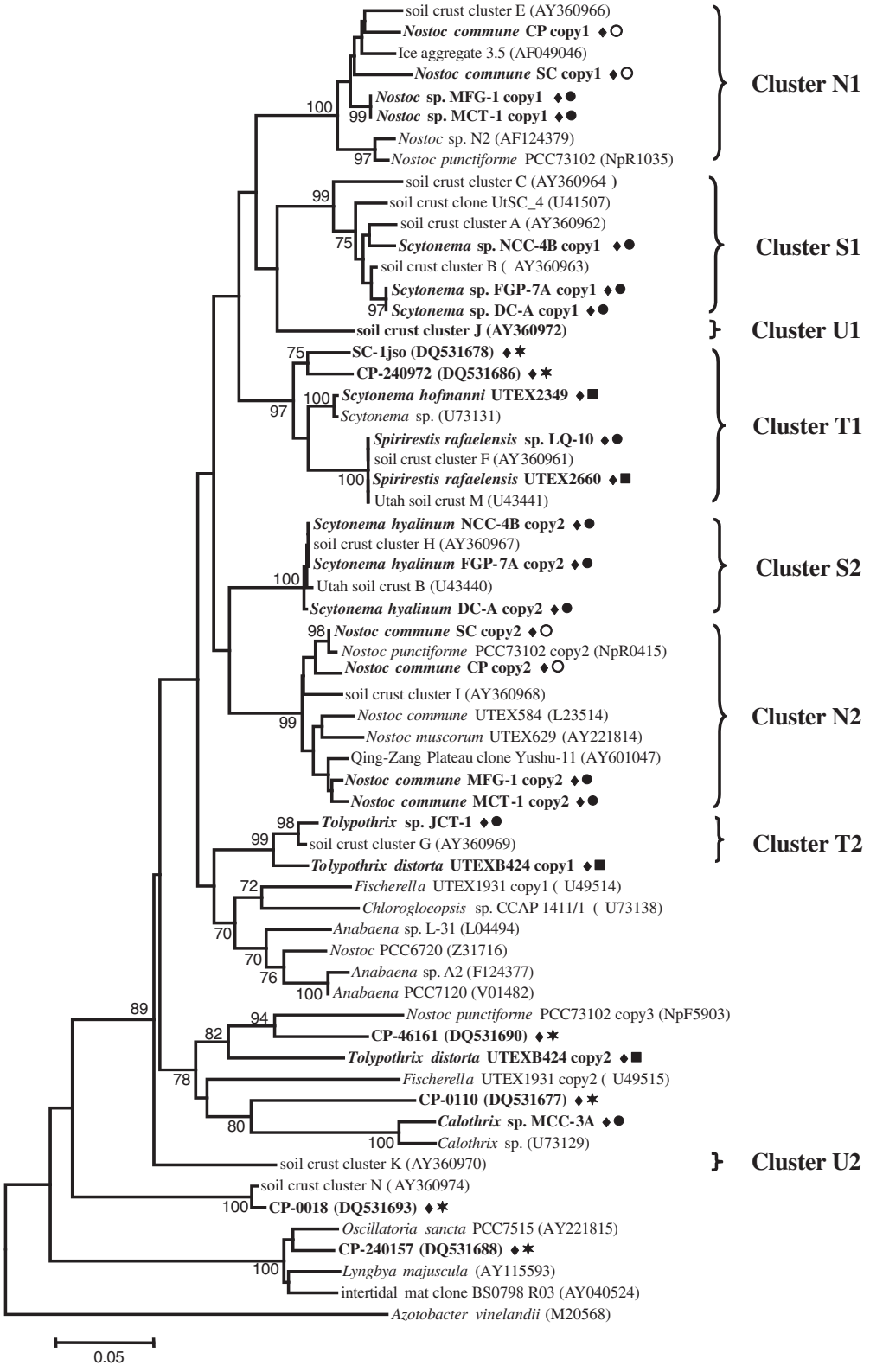

Table 2. Source and sequence (*nifH* and 16S rRNA gene) information for cyanobacterial strains

Isolate*	Sampling location†	Source material	*nifH* cluster‡	GenBank accession #	
				nifH gene	16S rRNA gene
Nostoc commune SC	SC1	*N. commune* colony (black, leaf-shaped masses)	N1, N2	DQ531670 DQ531672	DQ531705
Nostoc commune CP	CP2	*N. commune* colony (black threads)	N1, N2	DQ531679 DQ531671	DQ531706
Nostoc commune MCT-1 (CY05)¶	CP1	*Collema tenax* (lichen) thallus	N1, N2	DQ531680 DQ531689	DQ531703
Nostoc commune MFG-1 (CY06)	Moab	crust	N1, N2	DQ531687 DQ531683	DQ531699
Scytonema hyalinum FGP-7A	Moab	crust	S1, S2	DQ531669 DQ531674	DQ531698 DQ531697
Scytonema hyalinum DC-A (CY16)	CP1	crust	S1, S2	DQ531695 DQ531691	DQ531701 DQ531704
Scytonema hyalinum NCC-4B (CY18)	CP4	crust	S1, S2	DQ531694 DQ531675	ND§
Spirirestis rafaelensis LQ-10 (CY17)	CP4	crust	T1	DQ531673	DQ531696
Tolypothrix JCT-1 (CY19)	JO1	crust	T2	DQ531682	DQ531702
Calothrix MCC-3A (CY15)	CP1	*Collema coccophorum* (lichen) thallus	NC	DQ531692	DQ531700
Tolypothrix distorta UTEXB424	NR	potting soil	T2, NC	DQ531681 DQ531676	ND§
Spirirestis rafaelensis UTEX2660	SRS	crust	T1	DQ531685	AF334690–AF334692
Scytonema hofmanni UTEX2349	NY	Rock surface of walking trail	T1	DQ531684	AF132781

**N. commune* SC and *N. commune* CP designate macrosopic colonies of *N. commune* rather than bacterial isolates.

†SC1, Sangre de Cristo Mountains near Santa Fe, NM, USA; CP1 & CP2, Island in the Sky region of Canyonlands National Park, UT, USA; CP4, Needles region of Canyonlands National Park, UT, USA; JO1, Jornada Research Range, Chihuahuan Desert, NM, USA; Moab, near Moab, UT, USA; NR, Utrecht, the Netherlands (Starr & Zeikus, 1987); SRS, San Rafael Swell of the Colorado Plateau, UT, USA (Flechtner, 2002); NY, Watkins Glen State Park, NY, USA (Starr & Zeikus, 1987).

‡*nifH* Sequences (310 bp) were grouped into clusters based on visual inspection of alignments, distance data, and NJ trees. Sequences within each cluster were at least 95% similar (most were > 98% similar); NC, no cluster. Most isolates contained multiple *nifH* copies, representing different clusters.

§The 16S rRNA gene sequence for *S. hyalinum* NCC-4B was not deposited (it was identical to that of *S. hyalinum* DC-A). The 16S rRNA gene sequence for *Tolypothrix distorta* UTEX B424 was not determined.

¶Numbers in parentheses are culture archive numbers.

Morphological and molecular (nifH and 16S rRNA gene) analysis of cultured, N_2-fixing cyanobacteria

To identify the bacterial species harboring the various cyanobacteria *nifH* sequence-types, N_2-fixing cyanobacteria were cultured from Colorado Plateau and Chihuahuan Desert crusts. Morphotypes resembling bacteriological descriptions (Boone & Castenholz, 2001) of *Nostoc*, *Calothrix*, *Scytonema*, and *Tolypothrix* species were obtained in unicyanobacterial cultures (Table 2, Fig. 2).

Nostoc

Nostoc species were readily cultured from crust samples, lichen tissues (*Collema tenax* or *Collema coccophorum*), and from macroscopic colonies of *N. commune* (brittle, black, leaf-shaped masses and brittle, black threads) that were conspicuously found on the soil surface of the Colorado Plateau. On BG-11$^-$ agar plates the *Nostoc* strains formed dark green ovoid colonies or 'pearls' (Martinez & Querijero, 1986). Vegetative spherical cells of the *Nostoc* sp. were typically 3–5 μm in diameter, and various developmental stages including hormogonia, vegetative chains, and sheathed, filament primordia could be observed (Fig 2a).

The diversity of 16S rRNA gene sequences obtained from the 12 *Nostoc* strains cultured in our laboratory is represented by the sequences from strains *N. commune* MCT-1 and *N. commune* MFG-1 and from sequences obtained directly from macroscopic leaf (*N. commune* SC) and thread (*N. commune* CP) colonies of *N. commune* (Table 2). The 16S rRNA gene sequences from the *Nostoc* strains shared 96–100% (400 bp) similarity and all fell within *Nostoc* clade II as described by Svenning *et al.*(2005). The 16S rRNA gene

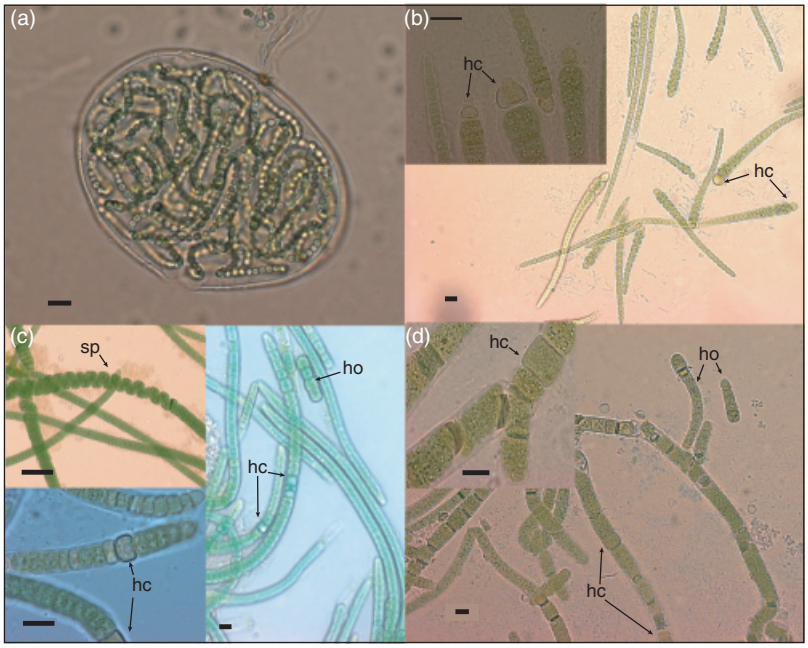

Fig. 2. Morphotypes of cyanobacterial isolates from biological soil crusts. (a) *N. commune* MCT-1 cells in filament primordia. (b) *Calothrix* sp. MCC-3A with terminal heterocysts (hc, arrows), (c) Disk-shaped cells in filaments of *Tolypothrix* sp. JCT show mild apical tapering; spiraling filaments (sp) were observed in *S. rafaelensis* LQ-10 (upper left insert); heterocysts (hc). (d) *S. hyalinum* FGP-7A exhibiting false branching, heterocysts (hc), and hormogonia (ho). Scale bars in each figure represent 10 μm.

sequences from the crust strains were most closely related to numerous environmental clones representing lichen symbionts and several cultured species of *N. commune*.

Two copies of the *nifH* gene were consistently amplified from each of the *N. commune* isolates. Sequence analysis placed the copies into *nifH* Clusters N1 and N2 (Fig. 1). The completely sequenced genome of *Nostoc punctiforme* PCC 73102 (isolated from a root section of a *Macrozamia* sp. in Australia) reveals that this organism has three *nifH* copies, one that falls within Cluster N1 (NpR1035), one that falls within Cluster N2 (NpR0415), and one that is not closely related to currently deposited *Nostoc nifH* sequences (NpF5903) (Fig. 1). From these results, we conclude that *nifH* sequences within Clusters N1 or N2 typically belong to *N. commune* and closely related strains within the species.

The ratio of N1 to N2 *nifH* sequence-types obtained from the environmental clone libraries was *c.* 4 : 1 (Table 1). A similar disparity was observed with the ratio of N1 : N2 sequences obtained from *N. commune* isolates (data not shown). These results suggest either that the N1 sequence-type is present in multiple copies in *Nostoc*, or that the PCR amplification or cloning efficiency for the N1 sequence-types was greater than that for the N2 sequence-types.

Scytonema

Scytonema species were also commonly observed on BG-11$^-$ agar plates inoculated with crust material, often forming phototrophic, aerial 'tufts' raised above the agar surface. Older cultures appeared yellow/brown, probably due to synthesis of the photo-protective pigment, scytonemin (Garcia-Pichel *et al.*, 1991; Castenholz & Garcia-Pichel, 2000). Cells within vegetative filaments typically ranged from rectangular to cylindrical in shape and measured 5–15 μm in length and 6–10 μm in width. False branching and heterocysts (primarily intercalary) were commonly observed (Fig. 2d). The morphological characteristics of our *Scytonema* strains are consistent with the description of *Scytonema hyalinum* Gardner provided by Flechtner *et al.* (2002).

Replicated PCR, cloning, and sequencing attempts with the *Scytonema* strains always yielded two divergent 16S rRNA gene copies (copy1 and copy2, which are 91% similar over 747 bp). One of these 16S rRNA gene copies (copy1) is identical (424/424 bp) to the 16S rRNA gene sequences from *Scytonema hyalinum* FI-8A and *Scytonema* cf. *javanicum* CCMEE5099, strains that were isolated from a Mojave Desert soil sample (Flechtner *et al.*, 2002) and a building wall in Bermuda (www.cultures.uoregon.edu), respectively. The other 16S rRNA gene copy identified in *Scytonema hyalinum* FGP-7A is not closely related to the 16S rRNA gene from other cultured strains (closest relative is *Anabaena bergii* 283A; 523/563 bp, 92%), but it is identical (378/378 bp) to 16S rRNA gene environmental clone sequences obtained previously from crusts of Canyonlands National Park in the Colorado Plateau region (Redfield *et al.*, 2002).

It could be argued that the two 16S rRNA gene copies found in these *S. hyalinum* strains are indicative of a coculture of morphologically identical species or that one

of the 16S rRNA gene copies belongs to a minute strain not easily detected by standard microscopy. However, several lines of evidence led us to conclude that the two 16S rRNA gene copies identified in these *S. hyalinum* isolates represent divergent intra-genomic operons. First, individual filaments of *S. hyalinum* FGP-7A were picked and dragged across the agar surface multiple times to obtain unicyanobacterial and clonal (genetically homogeneous) cultures independently in the Garcia-Pichel and Kuske laboratories. Second, *S. hyalinum* FGP-7A appeared unicyanobacterial under both the dissecting scope and microscope (up to $\times 1000$ magnification). Third, the same two 16S rRNA gene copies were also obtained from strains *S. hyalinum* NCC-4B and *S. hyalinum* DC-A, which were independently isolated from crusts collected at different sites in the Colorado Plateau region (Table 2).

Scytonema isolates from three distinct locations in the current study (*S. hyalinum* FGP-7A, *S. hyalinum* NCC-4B, and *S. hyalinum* DC-A; see Table 2) each contained two copies of *nifH* that grouped into Cluster S1 or S2 (Fig. 1). From the sequencing and morphological analysis, it is reasonable to conclude that *nifH* sequences that group within Clusters S1 or S2 belong to a group of cyanobacteria represented by *Scytonema hyalinum*-type strains.

Tolypothrix and *Spirirestis*

Morphotypes resembling *Tolypothrix* or *Spirirestis* species were observed much more infrequently in the Colorado Plateau crust BG-11$^-$ enrichments than the other genera. In fact, only one such culture (*Spirirestis rafaelensis* LQ-10) was obtained from these enrichments. However, during parallel BG-11$^-$ enrichments using Chihuahuan Desert crust material (Site JO1) many *Tolypothrix* morphotypes were observed and cultured (represented by *Tolypothrix* JCT-1). Trichomes of both *Tolypothrix* and *Spirirestis* strains were often slightly tapered at the ends, exhibited false branching, and contained primarily apical, but also intercalary heterocysts (Fig 2c). Individual cells were cylindrical to disk-shaped and were typically 6–11 μm wide and 4–6 μm long. A spiral morphotype, as observed for *S. rafaelensis* by Flechtner *et al.*(2002), was observed when cells of *S. rafaelensis* LQ-10 (but not *Tolypothrix* JCT-1) were grown in BG11 media containing $NaNO_3$ (Fig. 2c).

Partial 16S rRNA gene sequences obtained from *S. rafaelensis* LQ-10 and *Tolypothrix* JCT-1 were 99% similar (734 bp) to each other and shared 99–100% similarity (426 or 402 bp) with 16S rRNA genes from both *S. rafaelensis* SRS6 (also known as UTEX 2660; isolated from a crust sample collected from the San Rafael Swell of the Colorado Plateau) and *Tolypothrix distorta* SEVs-5-2CA (isolated from Chihuahuan Desert soil; Sevilleta LTER, New Mexico) (Flechtner *et al.*, 2002). Along with 16S rRNA gene sequences from *Coleodesmium wrangelii* MC-JRJ1 (isolated from a creek in Great Smoky Mountains National Park, Tennessee), these *Tolypothrix* and *Spirirestis* 16S rRNA gene sequences form a clade that Flechtner *et al.* classify as belonging to the botanical family *Microchaetaceae* (Flechtner *et al.*, 2002).

A single *nifH* copy was amplified from *S. rafaelensis* LQ-10, which grouped into Cluster T1 and was identical to several environmental crust *nifH* clones identified in previous Colorado Plateau surveys (Steppe *et al.*, 1996; Yeager *et al.*, 2004). The single *nifH* sequence identified in *Tolypothrix* JCT-1 grouped into Cluster T2 and was similar (99%) to that of environmental *nifH* clones obtained from Chihuahuan Desert crusts (Yeager *et al.*, 2004). To compare the *nifH* sequences obtained from *S. rafaelensis* LQ-10 and *Tolypothrix* JCT-1 with those from closely related species, we obtained *S. rafaelensis* UTEX 2660 and *Tolypothrix distorta* UTEX B424 for *nifH* analysis. The single *nifH* gene amplified from *S. rafaelensis* UTEX 2660 was identical to that from *S. rafaelensis* LQ-1. Two *nifH* copies were obtained from *T. distorta* UTEX B424: one that was 96% similar to that from *Tolypothrix* JCT-1 and a second that was determined to be a cyanobacteria-type *nifH*, not closely related to other published sequences ($< 90\%$). From these results, we infer that Colorado Plateau crust bacteria harboring *nifH* Cluster T1 sequences typically belong to *S. rafaelensis* of the *Microchaetaceae* family (Flechtner *et al.*, 2002). Most environmental *nifH* clones that were retrieved from crusts of the Colorado Plateau and classified as belonging to Cluster T1 were highly similar ($> 98\%$) to the *nifH* sequences from *S. rafaelensis* LQ-10 and *S. rafaelensis* UTEX 2660. However, a small percentage ($< 10\%$) of the T1 *nifH* sequences, such as SC-1jso and CP-240972, were not identical to the *Spirirestis* group and represent a different sequence type within the Cluster T1 (Fig. 1).

Calothrix

Calothrix morphotypes were easily obtained from the Colorado Plateau BG-11$^-$ enrichment cultures. Colonies formed wavy filaments on the agar surface with abundant amounts of opaque mucilaginous material visible. The filaments yellowed with age. The trichomes exhibited classic *Calothrix* morphology (Fig. 2b), and the strains produced motile hormogonia. The 16S rRNA gene sequence obtained from *Calothrix* MCC-3A was 99% similar (317 bp) to the 16S rRNA gene from *Calothrix parietina* SRS-BG14, which was previously isolated from the San Rafael Swell of the Colorado Plateau (Flechtner *et al.*, 2002), and 98% similar (645/657) to the 16S rRNA gene of *Calothrix desertica* PCC 7102, which was isolated from 'fine desert sand' near La Portado, Antofagasta, Chile. These sequences clustered tightly with

16S rRNA gene sequences from other *Calothrix* species (data not shown).

A single *nifH* copy was identified in *Calothrix* MCC-3A, one that did not group into any of the clusters designated in Fig. 1. The *nifH* sequence obtained from *Calothrix* MCC-3A was closely related (96%) to that from *Calothrix* sp. ATCC 27901 (the same strain as *C. desertica* PCC 7102). Thus, both the *nifH* and 16S rRNA genes from *Calothrix* MCC-3A are closely related to those from *C. desertica* PCC 7102, indicating that this strain (or close relatives) is widely distributed in arid soils. The next closest relative showed less than 85% identity with the *nifH* sequence from *Calothrix* MCC-3A. *Calothrix*-type *nifH* sequences were never detected in our environmental *nifH* gene surveys. These results are consistent with previous observations that neither *Calothrix* morphotypes nor *Calothrix*-type 16S rRNA gene sequences are numerically prominent in crusts of the Colorado Plateau (Garcia-Pichel *et al.*, 2001; Redfield *et al.*, 2002; Yeager *et al.*, 2004; Gundlapally & Garcia-Pichel, 2006) or Sonoran Desert (Nagy *et al.*, 2005). Although readily cultured from Colorado Plateau crusts, *Calothrix* species are most likely minor members of the region's crust diazotrophic community.

Discussion

The identity and diversity of the dominant diazotrophs in typical biological soil crusts of the Colorado Plateau were determined by combining large-scale environmental *nifH* surveys with morphological and molecular analysis of cultured cyanobacteria. N_2-fixing cyanobacterial strains containing *nifH* sequence-types that represented 89% (421/473) of the total *nifH* diversity found in the Colorado Plateau crust environmental clone libraries were obtained using simple media (BG-11 or BG-11$^-$) and a few permutations of temperature and light intensity. It was determined that the five major *nifH* sequence-types detected in direct *nifH* surveys of crusts (N1, N2, S1, S2, and T1) belong to three distinct phylogenetic groups:

(1) a group of *Nostoc* strains that belong to the morphospecies *N. commune*;

(2) a phylogenetically and morphologically coherent group of strains well-represented by isolates of the morphospecies *S. hyalinum*; and

(3) a phylogenetically coherent but morphologically less-defined group of strains that is represented by *S. rafaelensis*/*Tolypothrix* species.

Nostoc species are considered to be important components of the N_2-fixing community in nutrient poor, arid and semiarid soils worldwide (Dodds *et al.*, 1995; Potts, 2000; Wynn-Williams, 2000; Bhatnagar & Bhatnagar, 2005). Accordingly, *nifH* sequences closely related to the N1 and N2 sequence-types seem to be globally distributed. The N1 sequences from the *N. commune* strains isolated in this study were most closely related (94–99%) to *nifH* clones obtained from several arid and semiarid soil environments including Colorado Plateau biological soil crusts (Yeager *et al.*, 2004), soils from a mixed conifer forest in northern New Mexico (Yeager *et al.*, 2005), and soil particles blown into the permanent ice cover of Lake Bonney, Antarctica, from the surrounding McMurdo Dry Valley (Olson *et al.*, 1998). Based upon data from nitrogenase activity assays, small *nifH* surveys, and botanical field surveys of the surrounding soils, it was reasoned that *Nostoc* or *Nostoc*-like species were the dominant N_2-fixing bacteria in the Antarctic Dry Valley region and the Colorado Plateau (Olson *et al.*, 1998; Paerl & Priscu, 1998; Yeager *et al.*, 2004). From the data presented in the current study, it can be further surmised that the Antarctic and Colorado Plateau N1-type *nifH* sequences (and those identified in other studies) most likely belong to *N. commune*.

Sequences closely related (97–99% similar) to the S1 and S2 *nifH* clusters have only been identified in environmental clone libraries of biological soil crusts from the Colorado Plateau and Chihuahuan desert (Steppe *et al.*, 1996; Yeager *et al.*, 2004). All other *nifH* sequences share $\leq$ 92% similarity with the S1 and S2 sequence-types. From the available data, it is impossible to determine whether the S1 and S2 sequence-types are constrained to *S. hyalinum* and closely related species endemic to arid and semiarid regions of southwestern USA and northern Mexico, or this sequence type is under-represented because of the paucity of *nifH* sequences thus far obtained from arid land soils.

This study also identified *S. rafaelensis* as a potentially important N_2-fixing species in biological soil crusts of the Colorado Plateau. The *nifH* sequences of the two *S. rafaelensis* strains thus far isolated from the Colorado Plateau, LQ-10 and UTEX 2660, grouped together in Cluster T1 and were highly related to environmental *nifH* sequences recovered from the same region. In contrast, the *nifH* sequence recovered from *Tolypothrix* JCT-1 (isolated from Chihuahuan Desert soil crusts) grouped in Cluster T2 and was most closely related to environmental *nifH* clones from the Chihuahuan Desert. However, Cluster T2 also contains a *nifH* sequence obtained from a strain, *T. distorta* UTEX B424, which was isolated in 1948 from a flower pot in the Netherlands (Starr & Zeikus, 1987). Defining the relationships between *nifH* sequence-type, species and geographical distribution in the *Tolypothrix* and *Spirirestis* groups will require more detailed analysis of isolates and their genes.

Little is known about the ecophysiological factors that shape diazotrophic community structure and function in biological soil crusts; however, several traits of heterocystous cyanobacteria lend themselves to establishing these organisms as the dominant diazotrophs in crusts from arid and semiarid regions. First, it has been observed that N_2-fixing

cyanobacteria, particularly heterocystous species, generally exhibit greater resistance to dessication stress than do other groups of N_2-fixing bacteria (Stal, 1995; Rothrock & Garcia-Pichel, 2005; Yannarell *et al.*, 2006). Second, periods of microbial activity in soil crusts of many arid landscapes occur in short-term pulses (often measured in hours) when the soils are moist, and thus the underlying biogeochemical cycles of these regions are often subject to the ability of soil microorganisms to use pulses of resources (Austin *et al.*, 2004; Schaeffer & Evans, 2005). In cases where crust moistening occurs periodically during daylight hours (such as is often the case for the Colorado Plateau), N_2-fixing bacteria capable of quickly generating energy and fixing N_2 under the prevailing conditions (in the light) would have a strong competitive advantage. This constraint would preclude the strategy of separating periods of peak photosynthetic activity and nitrogen fixation temporally, as used by many nonheterocystous cyanobacteria and heterotrophs (Bebout *et al.*, 1987; Stal, 1995; Omoregie *et al.*, 2004). Indeed, we have observed that the nitrogenase activity (as measured by C_2H_2 reduction) of wetted Colorado Plateau crusts incubated in the light is typically five to 10 times greater than rates determined for samples incubated in the dark (data not shown). Finally, Garcia-Pichel *et al.* (Garcia-Pichel & Belnap, 1996a) found that the surface (upper 1–3 mm) of Colorado Plateau soil crusts becomes supersaturated with O_2 during peak periods of photosynthesis and they postulated that heterocystous N_2 fixation would most likely dominate under such high oxygen tensions.

Nostoc and *Scytonema* species are highly adapted for life in light intensive, arid environments. Through the production of scytonemin, mycosporines, and other photo-protective pigments these bacteria are able to shield themselves and, to a lesser extent, the surrounding microbial community from excessive UV irradiation (Castenholz & Garcia-Pichel, 2000). Accordingly, *Nostoc* and *Scytonema* species often inhabit the upper surface of biological soil crusts, maximizing their exposure to both light and episodes of transient moisture. Additionally, both of these microorganisms are resistance to dessication and cycles of freezing/thawing (Potts, 1994). It remains to be seen which of these qualities, or other traits, allow *S. rafaelensis* to thrive in the Colorado Plateau crusts, and whether the vertical distribution of this organism within crusts is similar or different from that of *Nostoc* and *Scytonema* species.

It has been hypothesized that a large fraction of N_2 fixation in Colorado Plateau crusts is facilitated by free-living heterotrophic bacteria or through a relationship between *Microcoleus vaginatus* and a N_2-fixing heterotroph (Steppe *et al.*, 1996; Billings *et al.*, 2003; Johnson *et al.*, 2005). Our results on soil crusts clearly do not support this hypothesis. However, they do not rule out a potential role for other N_2-fixing species in arid landscapes. Although the *nifH* Cluster O sequence type was less abundant ($< 10\%$) in the crusts than cyanobacterial *nifH* sequences, it may yet be an important component of the Colorado Plateau region crust diazotrophic community. Cultivation-independent surveys of bacteria in Sonoran (Nagy *et al.*, 2005), Colorado Plateau (Gundlapally & Garcia-Pichel, 2006), as well as Eastern Oregon crusts (Garcia-Pichel, unpublished) have shown that members of the *Oxalobacteria* (*Betaproteobacteria*) are common and widespread heterotrophic members in the communities, but it is not yet known whether any of these organisms are capable of N_2 fixation. In studies of microscale vertical distribution of N_2 fixation, Johnson *et al.*(2005) detected significant light-independent nitrogen fixation activity in the lower portions of Colorado Plateau crusts. It would be interesting to compare the spatial and temporal *nifH* gene expression patterns of the cyanobacterial and Cluster O *nifH* sequence-types within the crusts under varied conditions to determine whether these genes (and the species they represent) play functionally distinct or redundant roles in crust N dynamics.

One of the intriguing findings in this study was that the newly cultured *S. hyalinum* strains contained divergent (91% similar), intra-genomic 16S rRNA gene operons. Although the majority of prokaryotic 16S rRNA alleles within a single genome exhibit $< 1\%$ nucleotide variation, the occurrence of more divergent 16S rRNA alleles in a single bacterium is not without precedence. Acinas *et al.* (2004) found that five genomes, among 81 archaeal and bacterial genomes examined, harbored 16S rRNA alleles with higher than normal levels of sequence divergence (5.0–11.6%). Four of the genomes harboring divergent 16S rRNA alleles belonged to thermophilic bacteria, and the authors suggested that horizontal gene transfer may have been involved. Horizontal transfer of the 16S rRNA gene has also been reported to occur, albeit at a low frequency, among certain cyanobacterial taxa (Rudi *et al.*, 1998) and between cyanobacteria and proteobacteria (Miller *et al.*, 2005). Also among cyanobacteria, the presence of divergent operons in a single strain of *M. vaginatus* has been suspected, but not proven (Boyer *et al.*, 2002). The significance of multiple, divergent copies of the 16S rRNA gene in *S. hyalinum* FGP-7A and related strains, and the consequences of these findings on the interpretation of 16S rRNA-based analysis of cyanobacterial communities will require further research.

Detailed knowledge of the major species and their ability to perform key functions like N_2-fixation are essential to addressing outstanding questions in arid land ecophysiology and ecosystem functioning in response to changing environmental conditions. Environmental *nifH* surveys, calibrated using sequence and morphological information from cultured isolates, have more comprehensively identified the dominant N_2-fixing bacteria in soils from an arid

region. Similar approaches have been successful in beginning to unravel the relationships between community structure and function in other cyanobacteria-dominated environments (Nübel *et al.*, 2000; Abed *et al.*, 2002; Chacon *et al.*, 2005) and should be of more general applicability in microbial ecology, especially in light of the recent successes in culturing recalcitrant bacteria from the environment (Connon & Giovannoni 2002; Sait *et al.*, 2002; Stevenson *et al.*, 2004).

Acknowledgements

We would like to thank the JGI Sequencing Group at Los Alamos National Laboratory (LANL) for providing sequencing services, Stephanie Redman, Karri Langoni and Meghan Doyal for technical assistance, and Susan Barns and Shannon Johnson for helpful comments during manuscript preparation. This project was supported through a LANL Director's Funded Postdoctoral Fellowship and Savannah River National Laboratory's Mini-Sabbatical Fellowship to CMY, by grants to CRK and JB from the U.S. Department of Energy, OBER Program for Ecosystem Research, and by a grant to FG-P from the Soils Program of the USDA (NRI 35107-10054).

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